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# Oxidative stress in carcinogenesis. Correlation between lipid peroxidation and induction of preneoplastic lesions in rat hepatocarcinogenesis

Yesennia Sánchez-Pérez[a], Claudia Carrasco-Legleu[a], Claudia García-Cuellar[b], Julio Pérez-Carreón[a], Sergio Hernández-García[a], Martha Salcido-Neyoy[a], Leticia Alemán-Lazarini[a], Saúl Villa-Treviño[a,*]

[a]*Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV), Av. IPN No. 2508 Col. San Pedro Zacatenco, México 14, DF, CP 07360, Mexico*
[b]*Instituto Nacional de Cancerología, San Fernando No. 22, Tlalpan, México, DF 14080, Mexico*

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**Abstract**

Oxidative stress during carcinogen metabolism seems to participate in liver tumor production in the rat. *N*-diethylnitrosamine is an important carcinogen used in liver cancer animal models. This indirect alkylating agent produces DNA–ethyl adducts and oxidative stress. In contrast, *N*-ethyl-*N*-nitrosourea, a direct mutagen, which generates DNA–ethyl adducts, does not produce liver tumors in rat unless it is given under oxidative stress conditions such as partial hepatectomy or phenobarbital treatment. To gain insight into the relation between oxidative stress and hepatocarcinogenicity, the induction of preneoplastic liver lesions was compared among three different initiation protocols related to the initiation–promotion-resistant hepatocyte model. In addition, liver lipid peroxidation levels, determined as thiobarituric acid reactive sustances were studied early during the initiation stage. Rats initiated with *N*-ethyl-*N*-nitrosourea, 25 days after treatment developed fewer and smaller γ-glutamyl transpeptidase positive preneoplastic lesions than rats initiated with *N*-diethylnitrosamine. A pre-treatment with the antioxidant quercetin 1 h before *N*-diethylnitrosamine initiation, significantly prevented development of γ-glutamyl transpeptidase-positive lesions. Increased lipid peroxidation levels were induced with *N*-diethylnitrosamine from 3 to 24 h after initiation, while *N*-ethyl-*N*-nitrosourea did not induce increments, and importantly, pre-treatment with quercetin decreased lipid peroxidation induced by *N*-diethylnitrosamine. These results show correlation between lipid peroxidation and hepatocarcinogenicity and support the important role of oxidative stress on liver carcinogenesis.


*Keywords:* Oxidative stress; Initiation; Lipid peroxidation; Preneoplastic lesions; Hepatocarcinogenesis

* Corresponding author. Tel.: +52-55-5061-3993; fax: +52-55-5747-7081.
*E-mail address:* svilla@cell.cinvestav.mx (S. Villa-Treviño).

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## 1. Introduction

There are clear examples of the participation of reactive oxygen species (ROS) in hepatocarcinogenesis in the rat. Nakae et al. [1] showed that initiation with low doses of *N*-diethylnitrosamine (DEN) induced liver DNA-8-hydroxydeoxy-guanosine adducts and suggested that oxidative stress participates in hepatocarcinogenesis. Therefore, one can assume that initiation with high doses of DEN produces oxidative stress, as in the case of hepatocarcinogenic models such as that by Solt and Farber, initiated with DEN and promoted with 2-acetylaminoflourene (2-AAF) and partial hepatectomy (PH) as proliferative stimulus [2], or modifications of this, as in the Semple-Robert's [3] model. During a choline-deficient diet, the representative marker of oxidative DNA damage 8-hydroxy-deoxy-guanosin is induced [4] and the same is true after administration of cipofibrate, one of the more efficient peroxisome proliferators that induces liver cancer in the rat [5]. Trimethylarsine oxide, an organic metabolite of inorganic arsenics, produced liver tumors in male Fischer 344 rats and authors implicate a possible mechanistic role of oxidative DNA damage and enhanced cell proliferation [6]. Also, in male Fischer 344 rats, it was demonstrated that α,α-bis(*p*-chlorophenyl)-β,β,β trichloroethane (DDT) induces eosinophilic foci and hepatocellular carcinoma (HCC) as a result of oxidative DNA damage [7].

It is not clear yet if participation of oxidative stress depends on the DNA oxygen adducts or if there is a concomitant alteration of signalization by the abrupt induction of ROS during initiation, or if both, DNA damage and a new intracellular reduced steady state are necessary for carcinogenesis to take place. Evidence with U937 cells treated with two well-known nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and DEN showed that ROS are produced; these activate nuclear factor κB (NF-κB); subsequently, cyclooxygenase-1 (COX-1) activity is induced, and this pathway increases prostaglandin $E_2$ ($PGE_2$) synthesis [8]. These results, and evidence that acetylsalicylic acid, a non-steroidal anti-inflammatory drug, and NNK lung carcinogenesis inhibitors block activation of NF-κB, induction of COX-1 and $PGE_2$ synthesis, together lend support to the proposition that ROS participate in carcinogenesis. When NNK or DEN were substituted by their respective *O*-acetate derivatives, which do not need to be metabolized, they did not activate NF-κB or induce $PGE_2$ synthesis, even though it is known that these nitrosamines as well as their acetates produce DNA adducts [8,9].

There is evidence that oxidative stress is an obligatory component of carcinogenesis. It was recently communicated that COX-1 or COX-2-deficient mice had altered epidermal differentiation and, when treated with 7,12-dimethylbenz(a)anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-12,13-acetate (TPA), they presented reduced skin tumorigenesis, although DMBA stable DNA adducts were increased twice [10]. This study clearly shows that there is no correlation between adduct levels and tumorigenesis. Even though the authors do not demonstrate that oxidative stress is produced, a direct relation between COX-1 and COX-2 deficiency is hypothetically associated to a lesser degree of oxidative stress [10].

We propose that, in the rat hepatocarcinogenesis model, both direct alkylating DNA damage and alterations produced by ROS induction during carcinogen metabolism are necessary processes for liver cancer induction. The dilemma is how to differentiate the participation of DNA alteration by ethyl adducts [9] from the participation of cell modifications induced by ROS or, even more, from the obliged hypothetical participation of both to induce initiation. To gain insight into this problem, experiments were carried out to compare (a) induction of gamma-glutamyl transpeptidase-positive ($GGT^+$) preneoplastic lesions on the 25th day, as an early end point of hepatocarcinogenesis in a DEN-2AAF-PH model or (b) in the same model, substituting DEN by *N*-ethyl-*N*-nitrosourea (ENU), a direct carcinogen, that is only carcinogenic in the rat liver under very special circumstances [11–13] or (c) by administration of quercetin, an antioxidant, administered previous to the DEN, 2AAF treatment. And as a key comparative feature, in these three different groups, LPX was measured during the initiation period.

## 2. Methods

### 2.1. Reagents and animals

All reagents were purchased from SIGMA (St Louis, MO, USA). Male Fischer-344 rats

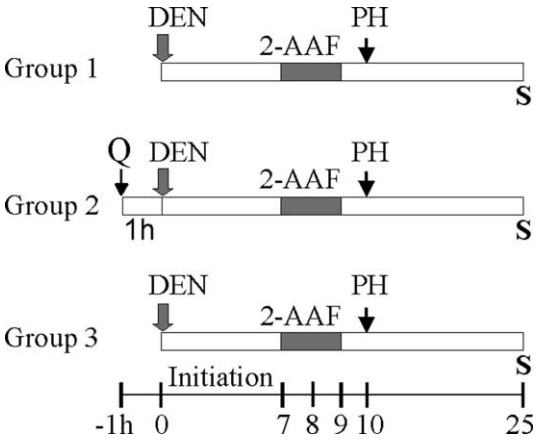


Fig. 1. Experimental design. Three groups of Fischer-344 rats were treated under three different conditions according to a modified Semple-Roberts hepatocarcinogenesis model. Group 1 ($n=30$) and 2 ($n=23$) received 200 mg/kg of intraperitoneal (IP) DEN as initiator. Group 2 received IP quercetin ($Q$) 50 mg/kg, 1 h before the 200 mg/kg of DEN. Group 3 ($n=23$) received 240 mg/kg of IP ENU. Sixteen rats of each group were sacrificed 3, 6, 12, and 24 h after initiation. Livers were homogenized and LPX determined. Finally, 14 rats from group 1, 7 from group 2 and 7 from group 3 received 20 mg/kg of oral 2-AAF from day 7 to day 10, in three daily consecutives doses, before partial hepatectomy (PH) at day10. These rats were sacrificed at day 25.

(180–200 g) were obtained from the CINVESTAV animal house, they had access to food (PMI Feeds, Inc., Laboratories Diet) and water at all times; food cups were replenished three times weekly. All animals received humane care and the study protocols were in compliance with the institutional guidelines for use of laboratory animals.

### 2.2. Experimental protocols

Two groups of rats were initiated with a dose of 200 mg/kg of DEN and a third group with a dose of 240 mg/kg of intraperitoneal ENU. All groups received 2-AAF orally, at doses of 20 mg/kg per day, during 3 consecutive days before PH. PH was performed 10 days after initiation. A third group was administered 50 mg/kg weight intraperitoneal quercetin dissolved in PBS–ethanol 10%, 1 h before DEN administration (Fig. 1). Animals were killed 3, 6, 12, 24 h and 25 days after initiation. Livers were excised, quickly frozen in liquid nitrogen and stored at −80 °C until analysis.

### 2.3. Quantitative analysis of $GGT^+$ preneoplastic lesions

Representative 20 μm thick sections from liver slices were obtained with a cryostat (Slee cryostat MTC, Germany) and stained for GGT activity according to Rutenburg et al. [14]. $GGT^+$ nodules were quantitatively analyzed using images of histological sections taken with a digital camera (ColorView 12, Soft Imaging System GmbH, Germany) coupled to stereological microscopy, and processed by an image software analysis kit (AnalySIS, Soft Imaging System GmbH, Germany). Focal $GGT^+$ areas greater than 0.05 $mm^2$ were registered to avoid small bile-duct cell detection (arbitrary intensity values) and were also classified according to GGT stain intensity. The number of preneoplastic lesions and their dimensions were scored in three sections/rat in 14 rats from group 1, 7 rats from group 2 and 7 rats from group 3. The data were analyzed by a two-tailed Student's $t$-test and $P$ values of 0.05 or lower were considered statistically significant.

### 2.4. Thibarbituric acid reactive substances (TBARS)

Samples of 0.8 g/ml frozen liver were homogenized in a buffer containing 10 mM Tris, 10 mM PMSF and 150 mM NaCl, pH 7.4; protein was determined according to Smith et al. [15]. LPX was measured as TBARS, a widely used assay, according to the method by Buege and Aust [16]. Briefly, 600 μg of protein of liver homogenates plus 300 μl of 0.8% of thiobarbituric acid (TBA) in 20% acetic acid, pH 3.0, were mixed and heated at 100 °C for 45 min. The samples were cooled, added with 200 μl of 1.2% KCl, 0.5 ml of 1:15 pyridine/butanol and centrifuged at 7500 rpm for 10 min. The absorbance of the resulting solution was determined at 532 nm, and TBARS were expressed with respect to malonyldialdehyde (MDA) by using the MDA extinction coefficient ($E=1.56\times10^5$).

## 3. Results

### 3.1. Preneoplastic lesions

Liver preneoplastic nodules were induced with a modified Semple-Roberts model [3], under three

different conditions as shown in Fig. 1. The $GGT^+$ lesions were measured at day 25 and our reference group initiated by DEN clearly presented abundant and high-intensity $GGT^+$ stained preneoplastic lesions while the other two groups of either ENU as initiator or quercetin plus DEN presented a few high-intensity $GGT^+$ stained preneoplastic lesions. However, all groups showed diffuse low-intensity $GGT^+$ stained areas in the liver (Fig. 2). A graphic representation of the area and amount of $GGT^+$ lesions is shown in Fig. 3. In group 1, high-intensity $GGT^+$ stained nodules occupied a total area of $5 \pm 3.3\%$, and the number of nodules was $31 \pm 6.4/cm^2$, while the group initiated with ENU presented a lesion-occupied area of only 0.6% and $2.6 \pm 1.4$ nodules/$cm^2$ and in the group with quercetin plus DEN, these values were $1.1 \pm 0.66\%$ in area and $16 \pm 0.44$ for nodules/$cm^2$.

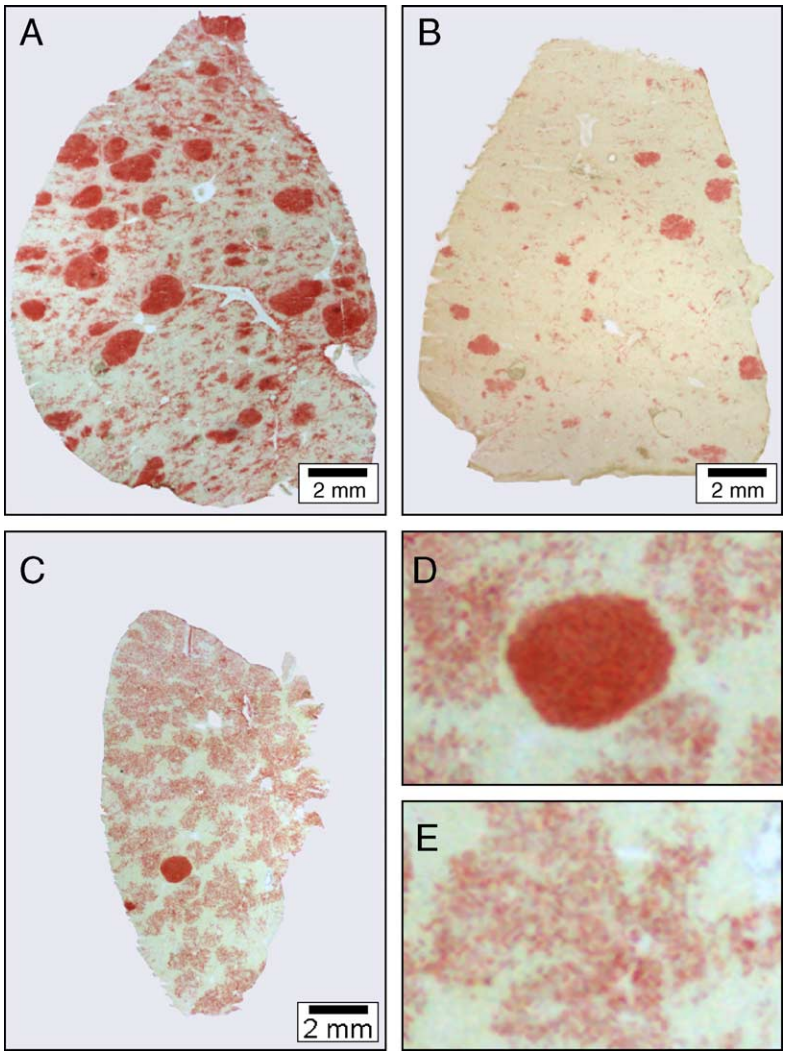


Fig. 2. Histochemical determination of $GGT^+$ lesions. Thick histological sections (20 μm) obtained from liver slices on the 25th day of treatment initiation were stained for GGT activity. Representative sections of each initiation protocol are shown. (A) Rat liver from group 1 initiated with DEN, (B) rat liver from group 2 which received 50 mg/kg IP quercetin 1 h before administration of DEN and (C) rat liver from group 3 initiated with ENU. Amplified images from C show high-intensity $GGT^+$ lesions (D) and low intensity $GGT^+$ lesions (E).

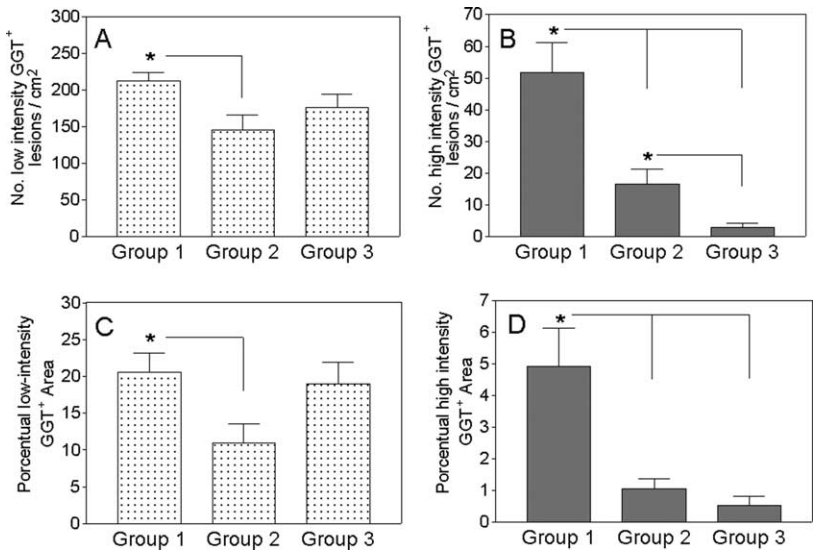


Fig. 3. Quantitative analysis of $GGT^+$ lesions in the initiation protocols. Focal $GGT^+$ areas larger than 0.05 $mm^2$ were registered and classified according to their low or high intensity by image analysis. The number of lesions and their dimensions were scored in three sections/rat. Group 1, liver section of rats initiated with DEN ($n=14$); group 2, liver of rats which received 50 mg/kg quercetin 1 h after being initiated with DEN ($n=7$); group 3, liver sections of rats initiated with ENU ($n=7$). Mean values $\pm$ SD. Statistical differences between groups are indicated by *.

### 3.2. Lipid peroxidation

Since it has been shown that LPX is an expression of oxidative stress in cells [17], we determined TBARS as a global approach and compared the levels obtained in a 24-h period after initiation, with the levels obtained during formation of preneoplastic lesions quantified 25 days after initiation. Three hours after initiation with DEN, an increment of LPX of 3.8-fold above control level was observed. The highest level, 4.62-fold, was obtained 12 h after DEN initiation, but all LPX determinations in this group at 3, 6, 12 and 24 h after DEN initiation showed statistically significant increments with respect to the vehicle control group (Fig. 4). Importantly, administration of quercetin, 1 h before DEN, prevented full expression of LPX at both, 3 and 6 h; represented only 15% and at 12 and 24 h, 67% and 66%, respectively, these values with respect to a similar group that did not receive quercetin. LPX levels at 3, 6, 12 and 24 h after initiation with ENU, instead of DEN, were very low compared with those obtained in non-treated animals and differences with respect to the DEN group were highly significant. The lowest of LPX levels induced with ENU, correlated with the induction of few preneoplastic lesions.

## 4. Discussion

To gain insight into oxidative stress participation in the induction of preneoplastic lesions, we analyzed

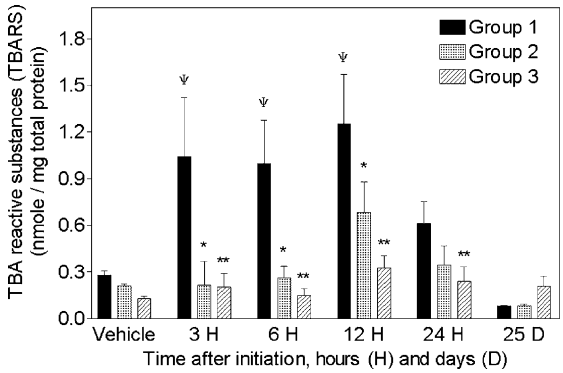


Fig. 4. Determination of lipoperoxidation after initiation with three different protocols. Malonyldialdehyde concentration as a result of LPX was determined in liver homogenates at 3, 6, 12 and 24 h and 25 days, after administration of initiator carcinogen (Fig. 1). Additionally, liver homogenates of rats treated with corresponding vehicles were used as controls. Water as DEN vehicle (group 1); 10% ethanol in PBS as quercetin vehicle (group 2); sodium citrate as ENU vehicle (group 3). Mean values $\pm$ SD. $\Psi$ $P$ values $\leq 0.03$ compared with their control vehicle. *$P$ values $\leq 0.03$ and **$pv \leq 0.001$ compared with group 1 at their respective times.

the effects during cancer initiation with ENU, a direct and seldom rat liver carcinogen [18] and with DEN an indirect and very efficient rat liver carcinogen, followed by 2AAF administration as a promoter and PH as proliferative stimulus. We chose to evaluate $GGT^+$ nodule formation at day 25 after initiation and LPX during 24 h after initiation. We show that DEN, a ROS-generating carcinogen [1] induces many preneoplastic lesions while ENU, a non-ROS-generating carcinogen, as it can be extrapolated from our result presented in Fig. 4, induces less preneoplastic lesions F. Also, administration of quercetin, an antioxidant [19], counteracted the oxidative stress induced by DEN and, as corollary of our results, the size and number of preneoplastic lesions closely correlated with the appearance of LPX.

Even though ENU has been shown to be a potent mono functional-ethylating and mutagenic agent in a variety of systems [20], its hepatocarcinogenic effect in rats seems to require previous PH [11,12] or to be followed with phenobarbital treatment [13]. In this respect, even intraportal administration to rats of 30 mg of ENU did not produce liver tumors [18]. ENU is used as a progressor in the initiation–promotion–progression model, late PH is performed and rats are sacrificed 6 months later, after which 12% incidence of HCC occurs in the PH rats and this incidence rises to 89% when PH is followed by PB treatment [13]. It should be noted that in both, PH [21,22] and PB [23] treatment, ROS are generated and PH strongly increases the mutagenicity or carcinogenicity induced by ENU [11,12,24]. These results agree with our proposal of the participative role of oxidative stress during hepatocarcinogenesis. In another context, Sakai et al. [25] investigated 26 chemicals in a model for detection of initiation activity. Among them they assayed *N*-methyl-*N*-nitrosourea (MNU), a carcinogen closely related to ENU, and showed that nitrosourea has activity as initiator. But in the used model, MNU was administered 24 h after hepatectomy, the period in which LPX increases to a maximum [22]. This evidence is in agreement with the hypothesis that the oxidative stress that occurs immediately during liver regeneration may contribute to initiator activity of MNU and by extension of other direct mutagens such as ENU [11–13].

In rats initiated with ENU, we can assume that liver DNA alkylation takes place, as has been shown by Frei et al. [26]. Nevertheless, in the hepatocarcinogenic model used herein, PH is performed 10 days after initiation, thus, ENU is not under the effect of the oxidative stress of HP. It may then be suggested that ENU alkyl-DNA lesions alone are not sufficient to induce preneoplastic lesions in rat liver. A similar conclusion can be reached in a multistage mouse skin model; in COX-1 or COX-2-deficient mice, which are hypothetically under a lesser oxidative stress, treatment with DMBA produced only 25% skin tumors, compared with wild-type mice, while stable DMBA–DNA adducts in COX-deficient mice increased two-fold, indicating again that the amount of DMBA–DNA adducts did not correlate with tumor number [10].

It has been accepted that the correlation between lipid peroxidation and appearance of preneoplastic lesions is indicative of ROS participation in carcinogenesis. It is tempting to speculate that alterations in Redox cell signaling produce a change that, synergically with DNA alterations, induces deregulation of cell control and hence cell proliferation. Such alterations have been shown in U937 cells treated with the carcinogens NNK and NDMA, which are metabolized by P450 cytochrome enzymes and produce oxidative stress, subsequently activate cyclooxygenases 1 and 2, leading to increase of $PGE_2$ and activation of NF-κB. In contrast, the *O*-acetates of the nitrosamines that do not require metabolism do not induce oxidative stress, or activate NF-κB or induce $PGE_2$ [8]. A possible mechanism of quercetin to reduce LPX levels and prevent preneoplastic lesion induction could be an inhibition of DEN metabolism capacity. Inhibition of carcinogen metabolism by quercetin has been previously reported [27]. Additionally, quercetin exhibits an antioxidant and free radical scavenging activities against $H_2O_2$ [28].

In summary, we presently show that liver LPX levels in rats initiated with DEN versus those initiated with ENU were several fold higher and differences were highly significant. This finding correlates with the virtual absence of liver preneoplastic lesions after initiation with ENU. Quercetin in our system blocks LPX and prevents preneoplastic lesion induction suggesting that the antioxidant effect participated in these phenomena. Our results support the proposal of ROS participation in the initiation of carcinogenesis.

The close correlation between the induction of liver LPX and the presence of preneoplastic lesions induced in the three different conditions studied with this hepatocarcinogenic model adds to the existing evidence of ROS participation in tumor induction and opens the possibility of further studies with this model to shed light on the mechanism of ROS participation in rat hepatocarcinogenesis.

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